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The Immune Response to a Vesicular Stomatitis Virus Vaccine Vector Is Independent of Particulate Antigen Secretion and Protein Turnover Rate

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Vesicular stomatitis virus (VSV) is a highly cytopathic virus being developed as a vaccine vector due to its ability to induce strong protective T cell and antibody responses after a single dose. However, little is known regarding the mechanisms underlying the potent immune responses elicited by VSV. We previously generated a VSV vector expressing the hepatitis B virus middle envelope surface glycoprotein (MS) that induces strong MS-specific T cell and antibody responses in mice. After synthesis in the cytoplasm, the MS protein translocates to the endoplasmic reticulum, where it forms subviral particles that are secreted from the cell. To better understand the contributions of secreted and intracellular protein to the VSV-induced immune response, we produced a vector expressing a secretion-deficient MS mutant (MS^{C69A}) and compared the immunogenicity of this vector to that of the wild-type VSV-MS vector in mice. As expected, the MS^{C69A} protein was not secreted from VSV-infected cells and displayed enhanced proteasome-mediated degradation. Surprisingly, despite these differences in intracellular protein processing, the T cell and antibody responses generated to MS^{C69A} were comparable to those elicited by virus expressing wild-type MS protein. Therefore, when it is expressed from VSV, the immune responses to MS are independent of particulate antigen secretion and the turnover rate of cytoplasmic protein. These results are consistent with a model in which the immune responses to VSV are strongly influenced by the replication cycle of the vector and demonstrate that characteristics of the vector have the capacity to affect vaccine efficacy more than do the properties of the antigen itself.

Vesicular stomatitis virus (VSV) is a nonsegmented negative-strand RNA virus belonging to the *Rhabdoviridae* family that causes vesicular lesions in cattle, horses, and pigs. Recombinant VSVs expressing foreign proteins have been studied as vaccine vectors for a number of pathogens, including HIV, influenza virus, hepatitis C virus, hepatitis B virus (HBV), measles virus, respiratory syncytial virus, severe acute respiratory syndrome virus, *Yersinia pestis*, papillomavirus, Ebola virus, and Marburg virus (10, 14, 15, 19–21, 28, 29, 51, 53, 56, 58). These vectors often generate protective cellular and humoral immune responses in a single dose. Though, in certain specific vaccination regimens, VSV is more effective at generating immune responses than are other potential vaccine vectors, including vaccinia virus (6, 15, 24), the mechanism by which it is able to elicit these superior responses is still largely unknown.

VSV is a highly cytopathic virus, which at high multiplicities of infection (MOIs) can cause cytopathic effects as early as 1 to 2 h postinfection. VSV replicates rapidly, resulting in the release of high numbers of progeny virus from infected cells. Virally expressed proteins are thus readily accessible to the major histocompatibility complex (MHC) class I pathway for the generation of a robust CD8 T cell response. Furthermore, though CD8 T cell-mediated killing is largely dependent upon intracellular antigen processing, CD8 T cell priming following VSV immunization could be due, in part, to cross-presentation of released antigen. Furthermore, those proteins released from infected cells may also be taken up by antigen-presenting cells (APC) and processed via the MHC class II pathway. In both the case of cross-presentation and that of MHC class II processing, it is unclear whether the rapid expression, processing, and secretion of viral proteins in infected cells contribute more to adaptive immune responses than do later VSV-induced cytopathic effects, which cause the release of viral antigens from infected cells and subsequent uptake by APC.

To further investigate the mechanisms by which VSV induces immune responses, we generated a vector expressing a secretion-deficient mutant of the HBV middle surface envelope glycoprotein (MS). Cellularly expressed wild-type MS proteins enter the endoplasmic reticulum (ER) and assemble to form particles, which can be secreted even in the absence of other HBV proteins (43). The mutant MS protein used in this study contains a cysteine-to-alanine alteration at residue 69 (C69A), which is known to be essential for subviral particle secretion (40).

In addition to secretion deficiency, previous work has shown that the MS^{C69A} mutant undergoes increased proteasomal degradation through the endoplasmic reticulum-associated degradation (ERAD) pathway (36). Increased proteasomal degradation of MS secretion-deficient mutants was also demonstrated to increase MHC class I epitope presentation and cytotoxic T lymphocyte (CTL) responses relative to those of wild-type MS in an *in vitro* assay (36), suggesting that targeting antigen for proteasomal degradation may be one strategy to enhance CTL responses to vaccination. Furthermore, recent studies of the HIV epitope repertoire revealed that antigen processing shapes CTL response hierarchies, suggesting that CTL responses to subdominant epitopes could be

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increased using similar strategies (60, 63). However, several studies counter that, in general, increased proteasomal degradation has no effect on CTL responses (22, 34, 65). Further work suggests that although increasing epitope presentation enhances CTL responses, once maximal CTL activity is reached, additional epitope presentation provides no further advantages (12, 66, 67). Using MS^{C69A}, we can therefore examine whether antigen processing and presentation following VSV immunization achieve maximal CTL responses via efficient epitope display.

We previously generated a VSV vector expressing wild-type MS (VSV-MS), which elicits robust HBV envelope-specific CD8 T cell responses and generates high antibody titers in mice (15). By comparing the cellular and humoral immune responses to HBV envelope following immunization with either VSV-MS or VSV-MS^{C69A}, we demonstrate here that the immune responses to VSV-expressed proteins are independent of particulate antigen secretion and the turnover rate of cytoplasmic protein. Our results are consistent with a model in which the potent T cell and antibody responses generated by VSV are due to certain properties of VSV replication and help us to understand why VSV can elicit superior immune responses compared to those of other potential vaccine vectors.

MATERIALS AND METHODS

Recombinant viruses. MS^{C69A} was amplified by PCR from pCMV-C69A (37) using primers 5′-CGTCGACATGCAGTGGAATTCCACAACC-3′ and 5′-GCTAGCTTAAATGTATACCCAAAGACA-3′, introducing upstream SalI and downstream NheI sites for directional cloning. The MS^{C69A} PCR product was cleaved with SalI and NheI and cloned into the fifth position of the pVSVXN2 plasmid after its cleavage with XhoI and NheI.

A recombinant VSV vector containing MS^{C69A} (VSV-MS^{C69A}) was recovered as previously described (35). Briefly, BHK-21 cells grown to 50% confluence were infected with recombinant vaccinia virus expressing T7 RNA polymerase (multiplicity of infection [MOI], 10) and incubated for 1 h in serum-free Dulbecco modified Eagle medium (DMEM). Vaccinia virus-infected cells were then cotransfected with the generated plasmid expressing the recombinant VSV antigenome and the VSV N, P, and L proteins under the control of a T7 promoter. Supernatants were collected 48 h posttransfection, filtered through an 0.2-μm filter to remove vaccinia virus, and passaged onto fresh BHK-21 cells. The medium was collected immediately after cytopathic effects were observed (~2 days) and filtered through an 0.1-μm filter. Recombinant VSV-MS^{C69A} was then plaque purified and grown, its titer was determined, and it was stored at −80°C until use. Recombinants were thawed and diluted to the correct titration immediately prior to use.

Recombinant VSV with no foreign insert (empty VSV) and a previously generated VSV expressing the ayw serotype middle envelope protein of HBV (VSV-MS) (15) were used in parallel with VSV-MS^{C69A}. Viruses encoding MS or MS^{C69A} and containing a deletion of a methionine at position 51 in the matrix protein (ΔM51) were similarly generated as previously described (46).

Detection of MS. BHK-21 cells were infected with VSV-MS or VSV-MS^{C69A} (MOI, 10) for 8 h. Four hours postinfection, cells were treated with 2 μM or 10 μM lactacystin (Calbiochem). Medium was collected, and cells were washed with phosphate-buffered saline (PBS) and lysed with 2× SDS sample buffer. A portion of each lysate was treated with 100 units endoglycosidase H (EndoH) (New England BioLabs) for 1 h at 37°C prior to electrophoresis. Proteins were separated on a 10% SDS gel, transferred to a nitrocellulose membrane, probed with anti-HBs antibody (Santa Cruz Biotechnology, Inc.), and detected with secondary antibody using chemiluminescence.

Secreted HBsAg was detected both *in vitro* and *in vivo*. To confirm the secretion deficiency of MS^{C69A} *in vitro*, BHK-21 cells were infected with VSV-MS or VSV-MS^{C69A} (MOI, 10) for 8 h. Murine bone marrow cells (BMC) were collected and cultured in RPMI (Gibco) complete medium (10% fetal bovine serum, 50 U/ml penicillin, and 2 mM L-glutamine) in the presence of murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/ml) and murine tumor necrosis factor alpha (TNF-α; 50 U/ml; PeproTech) for 4 to 7 days, with replacement of 75% of medium and cytokines every 3 days to remove dead cells. BMC were then infected with VSV-MS or VSV-MS^{C69A} (MOI, 10) for 6 h. Infected-cell lysates and media were collected, and HBsAg was measured using a commercially available HBsAg enzyme-linked immunosorbent assay (ELISA) kit (International Immunodiagnostics). Likewise, secretion deficiency was confirmed *in vivo* following intranasal (i.n.) infection of VSV-MS or VSV-MS^{C69A} (10^6 PFU in 25 μl PBS). Bronchoalveolar lavage (BAL) was performed on euthanized animals, and the collected BAL fluid was used to measure HBsAg by ELISA.

Virus replication *in vitro*. The replication rates of VSV-MS and VSV-MS^{C69A} were compared in BHK-21 cells (MOI, 10). The culture supernatants were harvested, and titers were determined using a standard plaque assay on BHK-21 cells.

Immunization protocols. Female CB6F1 mice, 8 to 10 weeks of age, were purchased from Charles River (Wilmington, MA), while 4-week-old, female C57BL/6-Prf1^{tm1Sdz} and C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed at the Yale University School of Medicine animal facilities, and experiments were performed in accordance with Yale Institutional Animal Care and Use Committee-approved procedures. Inhalational anesthetization was performed on mice with 30% (vol/vol) isoflurane (Baxter) diluted in propylene glycol prior to all immunizations. Single intranasal (i.n.) inoculations of 10^6, 10^4, or 10^2 PFU were administered in a 25-μl volume for VSV, VSV-MS, VSV-MS^{C69A}, ΔM51-MS, or ΔM51-MS^{C69A}. DNA immunizations were performed by intramuscular injection of pCMV-MS or pCMV-MS^{C69A} (100 μg) in 100 μl PBS.

ELISPOT assay. A gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay set (BD Biosciences) was used to quantify CD8 T cell activation following immunization. Briefly, the 96-well plates provided were coated overnight with purified anti-mouse IFN-γ antibody (1:200). After removal of capture antibody, plates were blocked for 2 h using DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 2 mM L-glutamine. Splenocytes were purified from mice at the peak of the primary CD8 T cell response (day 7 postimmunization) or during the memory phase of the CD8 T cell response (day 30 postimmunization). Splenocytes were passed through 70-μm strainers (BD Falcon) and treated with ACK lysing buffer (Lonza). After washing with Hanks balanced salt solution (Gibco), cells were suspended in complete DMEM (Gibco) (10% fetal bovine serum, 50 U/ml penicillin, and 2 mM L-glutamine) and seeded at 2×10^5 cells/well. The cells were stimulated overnight at 37°C with HBV- or VSV-specific peptides at a concentration of 20 μg/ml, with media used as negative controls. Plates were washed with PBS-Tween (0.05%, vol/vol) and incubated with the provided biotinylated anti-mouse IFN-γ antibody (1:250) for 2 h at 25°C. After washing, streptavidin-horseradish peroxidase (HRP) (1:1,000) was added to wells and incubated for 1 h at 25°C. Following the final washes, AEC chromogen substrate (BD Biosciences) was added to the wells and allowed to develop at 25°C for 20 to 40 min. The reaction was stopped with dH_2O, and the plates were allowed to air dry before spot-forming cells (SFC) were enumerated.

An IFN-γ ELISPOT assay was also used to quantify CD4 T cell activation. ELISPOT assay plates were prepared as described above. Because we previously had found the CD4 T cell response to MS to be greater at day 14 postinfection than at day 7 (data not shown), splenocytes for this assay were removed 14 days postinfection and treated with collagenase D (Roche) for 30 min at 37°C. Treated spleen tissue was then passed through 70-μm strainers (BD Falcon). For CD4 T cell purification, splenocytes were treated with ACK lysing buffer (Lonza), washed with Hanks bal-

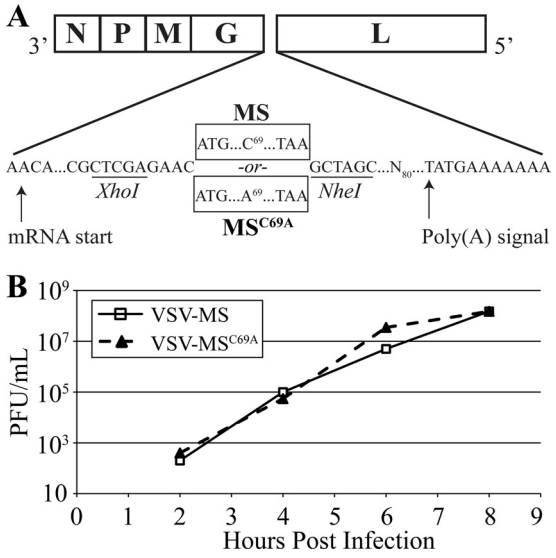

FIG 1 Generation and replication of VSV-MS^{C69A}. (A) The gene encoding MS^{C69A} inserted in the fifth position of the VSV genome, diagrammed from the 3′-to-5′ orientation of the negative-stranded viral RNA genome. (B) BHK cells were infected with VSV-MS or VSV-MS^{C69A}, and cell culture medium was collected 2, 4, 6, and 8 h postinfection. Standard plaque assays were performed to measure virus replication.

anced salt solution (Gibco), and suspended in complete DMEM. Cells were counted, centrifuged, and resuspended in magnetically activated cell sorting (MACS) buffer (Miltenyi). They were then incubated with 10 μl CD4 (L3T4) Microbeads (Miltenyi BioTec) per 10^7 cells for 15 min at 4°C. Cells were washed and resuspended in MACS buffer, and CD4 T cells were purified by positive selection using MACS separation columns (Miltenyi). Dendritic cells (DCs) were also purified from a separate set of prepared splenocytes. Splenocytes were centrifuged and incubated with CD16/32 antibody for 15 min on ice. The cells were then resuspended in MACS buffer and incubated with 100 μl CD11c Microbeads (Miltenyi BioTec) per 10^8 cells for 15 min at 4°C. Cells were washed and resuspended in MACS buffer, and CD11c DCs were purified by positive selection using MACS separation columns. Both CD4 T cells and CD11c DCs were resuspended in complete DMEM, counted, and seeded at 2×10^5 and 5×10^4 cells/well, respectively. Cells were incubated with 10 mg/ml recombinant MS protein (Biospacific) for 3 days at 37°C. Plates were developed as described above.

Antibody titers. HBV envelope-specific antibody (HBsAb) was detected in the serum using a commercially available HBsAb ELISA kit (International Immunodiagnostics). In order to determine the isotype-specific antibody present following immunization, diluted serum was incubated in a precoated HBsAg ELISA plate at 37°C for 60 min. The plate was then washed, and HRP-conjugated goat anti-mouse IgA, IgM, IgG1, or IgG2a (Santa Cruz Biotechnology, Inc.) was added and incubated at 37°C for 60 min. The plate was then washed, tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for 30 min, and the reaction was stopped using concentrated sulfuric acid.

RESULTS

Construction and characterization of recombinant VSV expressing HBV MS^{C69A}. The HBV middle surface (MS) envelope glycoprotein gene containing a cysteine-to-alanine mutation at residue 69 (MS^{C69A}) was PCR amplified and cloned into a plasmid DNA vector carrying the VSV genome (Fig. 1A). After sequencing confirmed that the plasmid contained no PCR-generated mutations, a recombinant VSV, designated VSV-MS^{C69A}, was recovered. *In vitro* replication of VSV-MS^{C69A} in BHK-21 cells was comparable to that of VSV-MS (Fig. 1B), indicating that overexpression of an aberrantly processed MS protein did not adversely affect virus replication.

An MS-specific antibody was used to characterize protein expression in VSV-MS^{C69A}-infected BHK-21 cells. Bands consistent with the molecular weight of single-glycosylated and double-glycosylated MS were detected in both VSV-MS- and VSV-MS^{C69A}-infected cells (Fig. 2A). Previous work demonstrated increased proteasomal degradation of the MS^{C69A} mutant by treating cells expressing the protein with the proteasome inhibitor lactacystin, which resulted in an increase in the unglycosylated form of the protein (36, 37). To determine if MS^{C69A} maintained this phenotype when expressed from a VSV vector, infected cells were treated with lactacystin, resulting in the increased accumulation of unglycosylated MS in VSV-MS^{C69A}-infected cells (Fig. 2B), as was previously described. We also determined whether MS^{C69A} maintained the secretion-deficient phenotype when expressed from VSV, which was observed in prior studies using DNA expression plasmids. Consistent with previous studies demonstrating that MS^{C69A} is improperly processed and/or trafficked intracellularly (36, 37), secreted MS protein was detected in the medium of VSV-MS-infected BHK-21 cells, but not VSV-MS^{C69A}-infected cells, by ELISA (Fig. 2C). Furthermore, secreted MS protein was detected in the medium of VSV-MS-infected GM-CSF-treated murine bone marrow cells (BMC), but not VSV-MS^{C69A}-infected BMC, confirming a similar phenotype for infected mouse immune cells. To verify that MS^{C69A} is also secretion deficient *in vivo*, mice were infected with VSV-MS or VSV-MS^{C69A} and BAL was performed. Unlike wild-type MS, mutant MS^{C69A} was not detectable at levels above background in the collected BAL fluid either 8 or 24 h postinfection (Fig. 2D), confirming that MS^{C69A} is secretion deficient when expressed from VSV *in vivo*.

VSV-MS^{C69A} elicits T cell responses comparable to those of VSV-MS. To compare the HBV-specific CD8 T cell response following VSV-MS^{C69A} immunization to that observed with VSV-MS, we conducted IFN-γ ELISPOT assays. At day 7 postimmunization, splenocytes were isolated from CB6F1 (H-2^{bxd}) mice receiving intranasal inoculations of 1×10^6 PFU of VSV, VSV-MS, or VSV-MS^{C69A}. The CD8 T cell response was analyzed following an overnight stimulation with a VSV N peptide (positions 52 to 59; RGYVYQGL), two peptides corresponding to known HBV-specific, H-2^d-restricted epitopes (HBs 191 to 202 [IPQSLD SWWTSL] and HBs 364 to 372 [WGPSLYSIL]), and two peptides corresponding to known HBV-specific, H-2^b-restricted epitopes (HBs 353 to 360 [VWLSVIWM] and HBs 371 to 378 [ILSPFLPL]) (4, 54, 55, 59). VSV-MS- and VSV-MS^{C69A}-immunized mice generated similar CD8 T cell responses to the four HBs peptides (Fig. 3A). As the magnitude, duration, and localization of antigen expression are also important for the establishment of long-lived, potentially protective T cell memory (5), we also measured the MS-specific memory T cell response by IFN-γ ELISPOT assay 30 days postimmunization. The memory responses were comparable between mice immunized with VSV-MS and those immunized with VSV-MS^{C69A} (Fig. 3B).

To determine if the secretion-deficient MS^{C69A} protein had an effect on the CD4 T cell response, IFN-γ ELISPOT assays were conducted using CD4 T cells purified from immunized animals. Mice were primed intramuscularly with 100 μg pCMV-MS or pCMV-MS^{C69A} and boosted 3 weeks later with intranasal inocu-

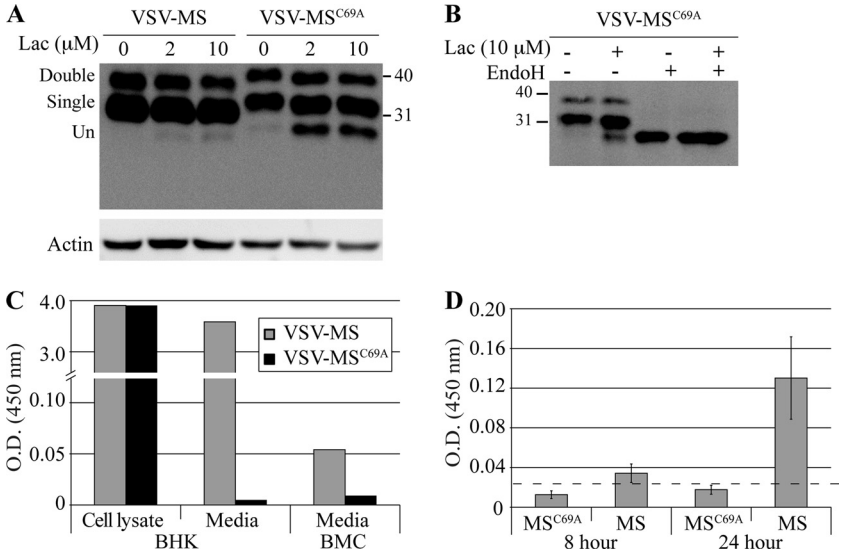

FIG 2 Characterization of MS^{C69A} expressed from VSV. (A) Western blot analysis with anti-HBsAg monoclonal antibody was used to assay for MS expression. BHK cells were infected with VSV-MS or VSV-MS^{C69A}, and 4 h postinfection, cells were treated with lactacystin or left untreated. Cells were harvested 8 h postinfection for analysis. Un, unglycosylated form; single, single-glycosylated form; double, double-glycosylated form. (B) Proteins in the lysate from VSV-MS^{C69A}-infected cells, both untreated and treated with lactacystin, were deglycosylated using an EndoH reaction. Numbers at right of panel A and left of panel B are molecular masses in kilodaltons. (C) Alternatively, infected BHK cell lysate and media as well as infected cultured bone marrow cell (BMC) media were collected 8 or 6 h postinfection, respectively. MS expression following infection was then determined by HBsAg ELISA. Background (measurements from empty VSV-infected cells) has been subtracted from all represented values. (D) At 8 ($n = 5$) and 24 ($n = 5$) hours after infection with VSV-MS or VSV-MS^{C69A}, BAL was performed on euthanized animals. An HBsAg ELISA was conducted to detect MS in the BAL fluid. The dashed horizontal line represents the background optical density value for the negative control in this assay (optical density at 450 nm = 0.027). All values are presented as the average of each group; error bars represent standard errors.

lations of 1×10^6 PFU of VSV-MS or VSV-MS^{C69A}, respectively. Fourteen days postboost, CD4 T cells were purified from the spleens. The CD4 T cell response was analyzed following a 3-day incubation with purified mouse dendritic cells and recombinant HBs protein. Surprisingly, VSV-MS and VSV-MS^{C69A} elicited comparable CD4 T cell responses 14 days postboost, despite the fact that MS^{C69A} was not properly processed or secreted when expressed from VSV (Fig. 3C).

VSV-MS^{C69A} elicits antibody responses comparable to those of VSV-MS. VSV-immunized mice normally generate high antibody titers to expressed antigen (15, 47). HBsAb ELISAs were conducted in order to determine the effect that expression of the secretion-deficient protein MS^{C69A} from VSV would have on antibody titers. Serum was collected from mice via retro-orbital bleed on days 30 and 60 post-intranasal immunization with 1×10^6, 1×10^4, or 1×10^2 PFU of VSV-MS or VSV-MS^{C69A}. There were no statistically significant differences between antibody titers measured on day 30 or 60 postimmunization in mice immunized with VSV-MS and those in mice immunized with VSV-MS^{C69A}, regardless of inoculum (Fig. 4A). As expected, we observed negligible measurable HBsAb titers in unimmunized mice (0.8 U/liter), consistent with our previous finding of an absence of MS-specific antibody titers in mice after immunization with empty VSV (15).

As secreted antigen and nonsecreted antigen released from a dead or dying cell may be processed and presented differently, there remained the possibility that the antibody responses to MS and MS^{C69A} may have developed differently. In order to rule out the possibility that processing and presentation differences may have resulted in differences in antibody class switching, an ELISA was conducted to determine the MS-specific antibody isotypes present following immunization with VSV-MS or VSV-MS^{C69A}. Despite differences in antigen processing, there were no statistically significant differences between MS-specific antibody titers for isotypes IgG1, IgG_{2a}, IgA, and IgM measured on day 30 postimmunization in mice immunized with VSV-MS or VSV-MS^{C69A} (Fig. 4B).

Decreased antibody response to MS^{C69A} following immunization with less cytopathic vectors. To confirm that antibody titers generated to the nonsecreted antigen MS^{C69A} were due to the potentially unique properties of VSV, antibody titers were also compared in mice immunized with the DNA vectors pCMV-MS and pCMV-MS^{C69A}. Mice received an intramuscular prime of pCMV-MS or pCMV-MS^{C69A} and were boosted with the same plasmids on days 30 and 60 postprime and bled on days 30, 60, and 90 postprime. In a DNA-based system, antigen secretion is required for antibody generation, as immunization with pCMV-MS generated significantly higher antibody titers ($P = 0.02$) than did that with pCMV-MS^{C69A}, which generated titers barely above the limit of detection (Fig. 5A). To further determine whether the cytopathic effects of VSV may be responsible for its superior ability to generate antibody responses, attenuated, recombinant VSV vectors containing a mutation in methionine 51 of the matrix protein (VSVΔM51) were generated. Unlike the wild-type recombinant VSV vectors, VSVΔM51 fails to inhibit interferon synthesis, resulting in early, high-level synthesis of IFN-α following infection. Infection with VSVΔM51 results in reduced cytopathic and apoptotic effects (27, 32). VSVΔM51-MS and VSVΔM51-MS^{C69A} vectors properly expressed HBV MS, which maintained secretion or secretion-deficient phenotypes, respectively, *in vitro* (data not shown). Mice received intranasal inoculations of 1×10^6

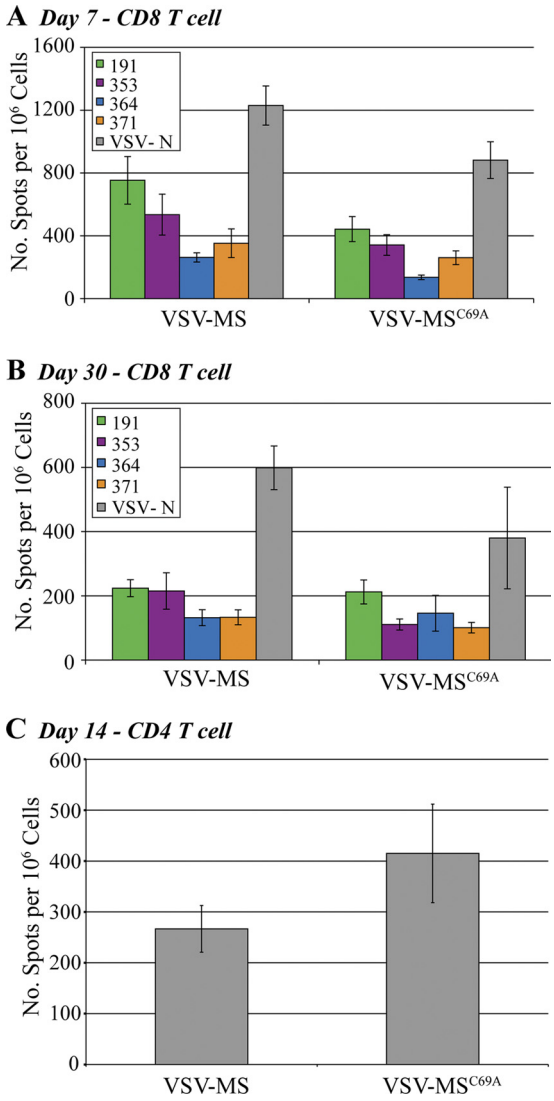

FIG 3 Specific T cell responses are elicited following a single immunization with VSV-MS. (A) CB6F1 mice were immunized with VSV-MS ($n = 9$) or VSV-MS^{C69A} ($n = 10$). Seven days postimmunization, splenocytes were harvested and analyzed using an IFN-γ ELISPOT assay. (B) A second set of CB6F1 mice was immunized with VSV-MS ($n = 8$) or VSV-MS^{C69A} ($n = 7$). Thirty days postimmunization, splenocytes were harvested and memory responses were analyzed using an IFN-γ ELISPOT assay. Two peptides corresponding to known HBV-specific, H-2^d-restricted epitopes (designated 191 and 364); two peptides corresponding to known HBV-specific, H-2^b-restricted epitopes (designated 353 and 371); and a VSV N peptide were used to stimulate splenocytes in the ELISPOT assays. (C) CB6F1 mice were boosted 3 weeks after pCMV-MS ($n = 3$) or pCMV-MS^{C69A} ($n = 4$) prime with VSV-MS or VSV-MS^{C69A}, respectively. Fourteen days postboost, CD4 T cells were purified and stimulated in an IFN-γ ELISPOT assay to measure the response. The number of cells responding to stimulation is represented as a quantification of the number of SFC/10^6 cells. All values are presented as the average of each group; error bars represent standard errors.

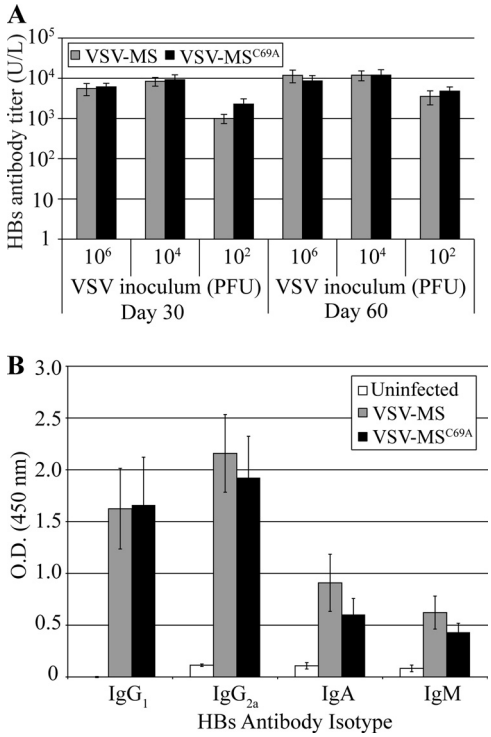

FIG 4 HBV-specific antibody responses following VSV-MS immunization are independent of MS secretion. (A) CB6F1 mice were immunized with various inocula (10^6, 10^4, or 10^2 PFU) of VSV-MS ($n = 15$) or VSV-MS^{C69A} ($n = 15$), and unimmunized mice were used as controls (0.8 U/liter; data not shown). A quantitative HBsAb ELISA was conducted on serum collected 30 and 60 days postimmunization to measure specific antibody titers. (B) CB6F1 mice were immunized with 10^6 PFU of VSV-MS ($n = 7$) or VSV-MS^{C69A} ($n = 8$); unimmunized mice were used as controls ($n = 7$). A qualitative HBsAb isotype-specific ELISA was conducted on serum collected 30 days postimmunization to determine isotype-specific antibody titers. Background (fetal bovine serum plus antibody) has been subtracted from represented values.

PFU of VSV-MS, VSV-MS^{C69A}, VSVΔM51-MS, or VSVΔM51-MS^{C69A}, and antibody titers were measured 30 days postimmunization. VSVΔM51-MS immunization resulted in significantly lower MS-specific antibody titers than did VSV-MS immunization (Fig. 5B), consistent with our hypothesis that the strong cytopathic effects of VSV contribute to the high antibody titers following immunization. However, because VSVΔM51 is also attenuated compared to the wild-type VSV vector, these results could also be due to more rapid control of VSV infection and therefore antigen expression, which may affect antibody responses in this system. Importantly, however, even lower antibody titers were generated after VSVΔM51-MS^{C69A} immunization than after VSVΔM51-MS immunization, further supporting the notion that in the absence of secretion, the cytopathic release of antigen is required for robust antibody responses in this system.

Antibody responses do not require CD8 T cell lysis. Both the direct cytopathic effects of VSV and the cytolytic T cell (CTL) response against the virus could contribute to the release of antigen from infected cells, thus explaining the high antibody titers observed following VSV immunization, even with the expression of a secretion-deficient form of antigen. To determine the contribution of the viral cytopathic effects relative to the cytolytic effects of the immune response, antibody titers to MS^{C69A} were measured in perforin-knockout mice. Perforin-knockout mice do not express perforin, a major component of the cytotoxic granules released from CTLs. Therefore, any release of virally expressed proteins in these mice can be attributed to the cytopathic effects of the virus itself rather than the cytotoxic immune response to infection. Wild-type (C57BL/6) and perforin-knockout (C57BL/6-

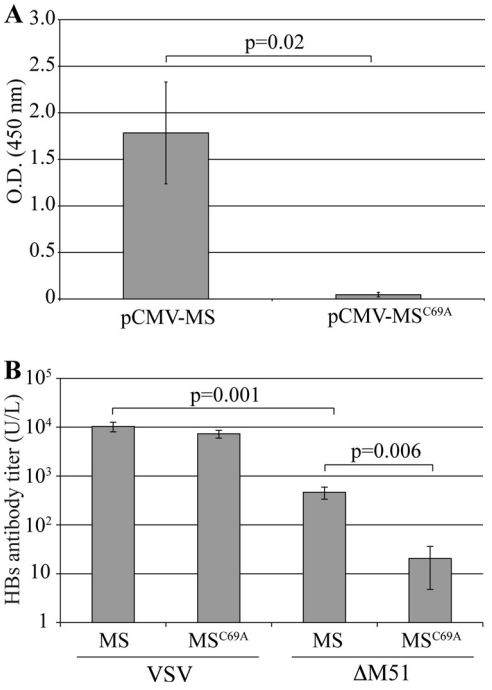

FIG 5 Reduced antibody responses to MS^{C69A} following immunization with DNA or attenuated VSV. (A) CB6F1 mice were primed with pCMV-MS (n = 7) or pCMV-MS^{C69A} (n = 5) and boosted with the same respective plasmid 30 and 60 days postprime. A qualitative HBsAb ELISA was conducted on serum collected 90 days postprime to determine specific antibody titers. (B) CB6F1 mice were immunized with 10^6 PFU of VSV-MS (n = 6), VSV-MS^{C69A} (n = 6), VSVΔM51-MS (n = 6), or VSVΔM51-MS^{C69A} (n = 6). A quantitative HBsAb ELISA was conducted on serum collected 30 days postimmunization to determine specific antibody titers. All values are presented as the average of each group; error bars represent standard errors.

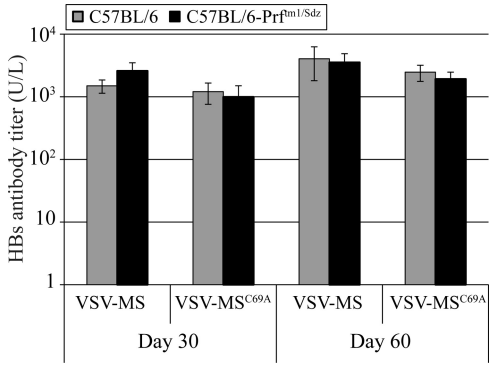

FIG 6 Perforin-dependent CTL-mediated killing does not contribute to antibody response. Perforin-knockout (C57BL/6-Prf1^{tm1Sdz}) (n = 8) and C57BL/6 (n = 10) mice were immunized with VSV-MS or VSV-MS^{C69A}. A quantitative HBsAb ELISA was conducted on serum collected 30 and 60 days postimmunization to determine specific antibody titers. All values are presented as the average of each group; error bars represent standard errors.

Prf1^{tm1Sdz}) mice were immunized with VSV-MS or VSV-MS^{C69A}. At both 30 and 60 days postimmunization, there were no statistically significant differences between antibody titers in wild-type mice and those in perforin-knockout mice immunized with either VSV-MS or VSV-MS^{C69A} (Fig. 6). This demonstrates that perforin-dependent CTL-mediated killing does not contribute to the antibody response following VSV immunization.

DISCUSSION

In certain experimentally tested vaccination regimens, VSV is more effective at generating immune responses than are other potential vaccine vectors, including vaccinia virus (6, 15, 23). However, the mechanism by which VSV is able to elicit these superior responses is still largely unknown. Attempts to significantly improve immunogenicity by altering the quantity or localization of antigen expression from VSV vaccine vectors have been generally ineffective (9, 14). This would suggest that VSV-based vaccine vectors contain intrinsic properties that serve to produce maximal immune responses to expressed antigen. In contrast to VSV-based vaccine vectors, modifying antigen expression in DNA-based vaccine vectors can alter their immunogenicity. Many groups have demonstrated that expression of degradation-targeted antigen from DNA vaccine vectors can improve CD8 T cell responses (11, 33, 36, 52). Antibody responses to DNA-based vaccines, however, are particularly sensitive to the properties of the expressed antigen. Studies of DNA-based vaccines expressing both *Tania ovis* and herpes simplex virus antigens have demonstrated that deletion of antigen signal sequences results in decreased antibody titers following immunization (17, 25). These data indicate that in a DNA-based vaccine system, antibody responses are dependent upon antigen secretion. This is further supported by a study in which antibody responses to HIV antigens expressed from DNA plasmids were increased when the genes were fused to a heterologous signal sequence from murine heavy-chain IgG (62). Furthermore, other studies have demonstrated that the alteration of antigen expression from a number of viral vaccine vectors can improve immune responses to vaccination. Targeting the localization of vectored antigen expression to dendritic cells is one strategy that has been utilized to improve immune responses following immunization with coronavirus-, lentivirus-, and adenovirus-based vectors (13, 38, 39). Vaccinia virus expressing endoplasmic reticulum (ER)-targeted minigenes has been demonstrated to improve CTL responses (49) and, in one study, elicited 10- to 1,000-fold-stronger responses in calcium mobilization, T cell receptor (TCR) downregulation, IFN-γ release, and T cell proliferation assays (57). Taken together, these studies suggest that, for a variety of vaccine vectors, different pathways may be responsible for generating the cellular and humoral responses, and thus, targeting these pathways can differentially affect the immune response following vaccination.

Here, we demonstrate that a VSV-based vaccine vector expressing a secretion-deficient HBV antigen (MS^{C69A}) induces HBV-specific immune responses comparable to those to the corresponding wild-type, secreted HBV antigen (MS). Despite the fact that the MS^{C69A} antigen is not secreted when expressed from VSV, VSV-MS and VSV-MS^{C69A} immunization generate similar CD4 T cell and antibody responses, which are dependent upon the MHC class II presentation pathway. Furthermore, although MS^{C69A} undergoes increased proteasomal degradation compared to wild-type MS, there is no difference in the CD8 T cell responses between vectors, though one might predict that increased cytoplasmic turnover of MS^{C69A} could enhance MHC class I presentation. These results are consistent with a model in which the potent T cell and antibody responses generated by VSV are due, at least in part, to maximal epitope production and virus-induced cytopathic effects, leading to antigen release and uptake by antigen-presenting cells.

VSV is a unique vaccine vector as it is a rapidly replicating, highly cytopathic virus, which, at high multiplicities of infection, can cause cytopathic effects as early as 1 to 2 h after infection. Both the viral glycoprotein and the viral matrix protein mediate the cytopathic effects of VSV. As the VSV glycoprotein has fusogenic properties, its expression results in the formation of syncytia between infected cells, promoting apoptosis (26). Furthermore, VSV matrix inhibits both host cell transcription and the transport of RNA from the nucleus to the cytoplasm (7, 8, 44). Using these mechanisms, matrix protein prevents host cell synthesis of type I interferon, allowing the virus to continue its replication cycle (2). The evasion of the host innate immune response by VSV likely provides antigen-presenting cells with an abundance of antigen as it is released from VSV-infected cells. This evasion may, subsequently, contribute to the superior adaptive immune response seen following VSV immunization.

Like other viral vectors, VSV provides a variety of "danger signals" that engage the host's Toll-like receptors (TLRs) and other pattern recognition receptors, activating the innate immune response and, thus, enhancing the adaptive immune response (30, 31). Viral vaccine vectors can, therefore, serve a dual function as both a recombinant protein expression vector and a vaccine adjuvant. The adjuvant properties of VSV are not sufficient, however, to induce the robust immune responses following VSV immunization. As viral vaccine vectors all express viral pathogen-associated molecular patterns (PAMPs) with adjuvant properties, the superior immune responses seen following VSV immunization may not be explained by its adjuvant properties alone and, instead, suggest that immunogenic differences could be attributed to differences in vector properties, including cytopathogenicity, protein expression levels, and temporal patterns, among others.

Previous work has demonstrated that intranasal inoculation with UV-inactivated VSV does not induce neutralizing antibodies to VSV or provide protection against viral challenge (50). Neutralizing antibody titers and protection against viral challenge can be achieved, however, with a single round of VSV replication (48). Together, these data also support a model in which antigen release is required for the robust immune responses observed following VSV immunization.

In our studies, we found that VSVΔM51, an attenuated vector with reduced cytopathic and apoptotic effects, expressing MS^{C69A} induces a lower MS-specific antibody response than does VSVΔM51-MS. Though the rapid control of VSV infection and, therefore, of antigen expression likely contributes to lower antibody titers in this system, as evidenced by the lower titers seen following VSVΔM51-MS infection than following VSV-MS infection, our findings support the hypothesis that generation of high antibody titers is dependent on the cytopathic effects of wild-type VSV. Furthermore, our findings are consistent with other studies of VSV matrix mutants. Infection with VSVΔM51 results in reduced cytopathic and apoptotic effects (27, 32). Interestingly, VSVΔM51 expressing a foreign protein, HIV Env (EnvG) (VSVΔM51-EnvG), is not capable of inducing as potent an EnvG-specific CD8 T cell response as that induced by immunization with wild-type VSV-EnvG (46). This difference in EnvG-specific CD8 T cell responses was observed for both intranasal and intramuscular immunization routes. It has also been demonstrated that poor antibody titers are generated following low-dose immunization with the VSV matrix mutant rM51R-M (1). Antibody titers were improved, however, by inserting flagellin into the rM51R-M vector, which is hypothesized to enhance DC function (3). Studies of attenuated VSV vectors in which viral cytopathic effects are preserved, such as single-cycle replication vectors, have demonstrated, however, that immune responses equivalent to those with wild-type vectors are achieved following intramuscular immunization (47, 48). Taken together, these studies further support the hypothesis that the induction of potent immune responses to VSV-expressed antigen following immunization is largely dependent on the viral cytopathic effects, antigen release, and subsequent uptake by APC.

Though CD8 T cell responses are largely dependent upon intracellular antigen processing, our results suggest that the superior CD8 T cell responses observed following VSV immunization could be due, in part, to cross-presentation of released antigen. The unique ability of VSV to induce rapid cytopathic effects may account for its superior induction of CD8 T cell responses compared to other viral vaccine vectors. This is further supported by a study demonstrating that targeting of antigen for rapid intracellular degradation can enhance CD8 T cell responses after immunization with a vaccinia virus vaccine vector (64). Our results would indicate that, if in fact cross-presentation is playing a role in the generation of the CD8 T cell responses that we observe following VSV immunization, VSV-induced cytopathic antigen release is more important in this pathway than is secreted antigen.

Alternatively, maximal CD8 T cell responses may be achieved following VSV infection due to rapid expression of viral proteins, allowing for optimal antigen processing and MHC class I peptide presentation. Many studies have demonstrated that although increasing epitope presentation enhances CTL responses, excessive epitope presentation does not provide additional advantages (12, 66, 67). This may provide an alternative explanation for the comparable CD8 T cell responses observed between VSV-MS and $VSV\text{-}MS^{C69A}$ immunizations.

A variety of viral vectors have been demonstrated to induce protective immune responses against a number of pathogens (16, 18, 41, 45, 61). Despite similarities in the principles of virus-based vaccination strategies, each vector can vary considerably in transgene expression, tissue tropism, and immunogenicity (42). The characteristics of the vector have the capacity, therefore, to affect vaccine efficacy as much as, if not more than, the properties of the vectored antigen itself. Our results support this finding and give insights into the superior immunogenicity of VSV as a viral vaccine vector. Our study indicates that the immune response to a VSV-based vaccine vector is independent of antigen secretion and cytoplasmic processing and suggests instead that the immune response is dependent upon the replication cycle of VSV. A better understanding of VSV-induced immunity will facilitate vaccine design and development for challenging and emerging pathogens.

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